Title: Calcium co-ingestion augments postprandial glucose-dependent insulinotropic peptide$_{1-42}$, glucagon-like peptide-1 and insulin concentrations in humans.

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Running title: Calcium co-ingestion: impact on GIP, GLP-1 and insulin.

ABSTRACT

Purpose This study determined whether calcium co-ingestion potentiates postprandial GIP_{1-42} and GLP-1 concentrations in humans, and the concomitant impact on insulin, appetite sensations and substrate metabolism.

Methods Ten healthy males consumed two energy- and macronutrient-matched meals in a double-blind, randomized, crossover design. The calcium content of the control meal was 3 mg/kg body mass, which was increased to 15 mg/kg body mass with calcium co-ingestion. Circulating concentrations of GIP_{1-42}, GLP-1 and insulin were determined over a 180-min postprandial period, followed by 60 min of exercise. Visual analogue scales were used to determine subjective appetite sensations. Rates of energy expenditure and substrate (lipid and carbohydrate) oxidation were estimated using indirect calorimetry.

Results Calcium co-ingestion resulted in a 47% increase in GIP_{1-42}, a 22% increase in GLP-1 and a 19% increase in insulin areas under the curve for the 120 min following consumption (all $P < 0.05$). Furthermore, appetite sensations were suppressed by calcium co-ingestion by 12% ($P = 0.034$). No differences, however, were observed in substrate metabolism ($P > 0.05$).

Conclusion Ingestion of a high-calcium meal potentiates postprandial GIP_{1-42}, GLP-1 and insulin concentrations in humans. Subjective appetite is also temporarily suppressed, although substrate metabolism is unaffected.

Keywords: *Dairy, GIP, GLP-1, Incretin, Appetite, Lipid oxidation, Exercise*

Abbreviations: GIP, glucose-dependent insulinotropic peptide; GLP-1, glucagon-like peptide 1; DPP-IV, dipetidyl-peptidase IV; NEFA, non-esterified fatty acid; VAS, visual analogue scale; CHO, carbohydrate; CON, control trial; CAL, high-calcium trial; AUC, area under the curve; RER, respiratory exchange ratio; VO_2, rate of oxygen consumption; VCO_2, rate of carbon dioxide production.

INTRODUCTION

Habitual calcium intake is inversely associated with obesity[1] and type 2 diabetes[2], and calcium and vitamin D supplementation can augment fat loss under energy restriction[3]. Currently, potential explanations for the protective effect of higher-calcium intake include, improvements in appetite regulation[4], increases in lipid oxidation[5] (which may be greater during exercise/energy deficit[6]) and/or reductions in dietary fat absorption[7].

Of these putative mechanisms, the least well studied is that of calcium intake and appetite. Some have shown a reduction in 24-h energy intake following a high-calcium meal [4], whilst others have found no difference in energy intake, appetite ratings, or postprandial concentrations of appetite-related hormones such as

insulin and glucagon-like peptide-1 (GLP-1)[8].

Following food consumption, the gastrointestinal peptides, glucose-dependent insulinotropic peptide$_{1-42}$ (GIP$_{1-42}$) and glucagon-like peptide-1$_{7-36}$ (GLP-1$_{7-36}$), are secreted by K-cells and L-cells of the intestine [for a review see Holst[9]]. These peptides potentiate insulin secretion by direct action on β-cells[10,11], but GLP-1$_{7-36}$ also acts via the nervous system[12,9] providing an anorectic component[13]. Due to their unique properties, therapies for obesity[14] and diabetes are currently being developed based on these peptides, with promising efficacy[15] thereby highlighting their metabolic importance. Further physiological effects of these peptides are currently being uncovered, including lipolysis[16] and substrate metabolism[17-20].

Both GIP$_{1-42}$ and GLP-1$_{7-36}$ provide a substrate for dipetidyl-petidase IV (DPP-IV) in the N-terminal regions, thought to be crucial for receptor activation[21,22]. Consequently, following cleavage, the remaining peptides GIP$_{3-42}$ and GLP-1$_{9-36}$ are thought to be principally inactive.

It is known that major macronutrients (fat and carbohydrate) stimulate GIP and GLP-1 secretion by direct contact with K- and L-cells[23]. Some recent evidence suggests that calcium could also play a crucial function. When isolated rodent intestine was perfused with increasing luminal concentrations of calcium [at humanly physiological concentrations[24]], total GIP (the accumulation of active and inactive forms) and GLP-1$_{7-36}$ secretion was stimulated[25]. The stimulation of GIP and GLP-1 by calcium was greater in the presence of the amino acid $_{L}$-phenylalanine indicating a synergistic effect. It is not currently known whether oral ingestion of calcium can augment circulating GIP and GLP-1 concentrations in humans. Due to the significance of GIP and GLP-1 in metabolic disease (whereby alterations in postprandial GIP and GLP-1 profiles are seen in type 2 diabetes[26] and improvements in glycemic control following bariatric surgery parallel changes in gut peptides[27]), they may help to explain the relationship between calcium intake, obesity and type 2 diabetes.

Accordingly the primary aim of the present study was to explore whether a co-ingestion of calcium with a meal augments gastrointestinal peptide concentrations in humans. Given the well-documented impact of these peptides on insulin secretion[10] and appetite[13], and some evidence that calcium can reduce energy intake[4], a secondary aim was to examine the effect of a high-calcium meal on insulinemia and appetite during the postprandial state. As both gastrointestinal peptides and calcium intake have been implicated in lipolysis[4,16] and substrate metabolism, particularly during exercise[6,20], the third objective was to assess the concomitant impact on substrate metabolism at rest and during exercise.

SUBJECTS AND METHODS

Participants

Young, healthy, recreationally active males were recruited between April-August 2012 from the student and staff population at Northumbria University. Participants provided written informed consent prior to the study. Eligibility criteria included, young (< 35 y), non-obese (BMI < 30 kg/m^2), self-reported physically active (>30 min of structured exercise, 5 times/week) and no known metabolic or gastrointestinal diseases or food allergies. The protocols were approved by the Faculty of Health and Life Sciences Ethics Committee at Northumbria University and are therefore in accordance with the Declration of Helsinki.

Preliminary measurements

Prior to main trials, participants undertook preliminary tests to establish 1) the relationship between oxygen uptake and running speed on a flat treadmill using a 16-min test, and 2) their VO_{2peak} using an incremental treadmill test whereby the gradient was increased by 1%/min to exhaustion as previously described in full detail[28]. On the same day, participants were familiarized with the visual analogue scales (VAS) to later assess subjective appetite sensations in main trials. A food frequency questionnaire, previously validated and used in similar populations[29,30], was completed to estimate habitual calcium intake.

Experimental design

Participants completed two trials in a randomized (randomization performed by J.T.G. using an electronic statistical package), double-blind (J.T.G. and the participants were blinded), crossover design separated by 7 d, which consisted of a control (CON) and high-calcium (CAL) trial (Figure 1).

All trials were performed under similar laboratory conditions (mean ± SEM; Temperature: 21.9 ± 0.7 and 21.9 ± 0.7 °C; Humidity: 39 ± 1 and 41 ± 2 %; Pressure: 1006 ± 2 and 1006 ± 4 mbar for CON and CAL trials, respectively; all $P > 0.05$).

Food and fluid diaries were kept for the day preceding the first trial and participants were instructed to replicate this for all subsequent trials. Participants were asked to avoid all foods containing dairy in the final meal prior to trials and to abstain from alcohol, caffeine and vigorous activity (defined as any structured

exercise) for 24 h prior to trials. Participants arrived at the laboratory at Northumbria University at 0730 after a 12 h fast.

Arterialized venous blood samples were obtained by catheterization of a pre-heated dorsal hand vein as previously described[31]. Following baseline VAS and blood samples, breakfast was consumed and consisted of, instant oats (Oatso Simple Golden Syrup, Quaker Oats, Reading, UK), whole milk (Cravendale, Arla Foods, Denmark) and 100 ml water. This was cooked in a microwave at 1000W for 2 min to produce a porridge of semi-solid consistency. This provided 0.5 g carbohydrate/kg body mass. (energy: 1258 ± 33 kJ; 299 ± 8 kcal, protein: 11 ± 0 g, carbohydrate: 41 ± 1 g and fat: 10 ± 0 g). We chose a mixed-macronutrient meal to exploit the synergistic dose-response between amino acids and calcium on GIP and GLP-1 secretion observed in rodent intestine[25], and the glucose-dependent insulin secretions of GIP and GLP-1[10].

The CON breakfast contained 3 mg calcium/kg body mass (248 ± 7 mg). The calcium content was increased for CAL trials to 15 mg calcium/kg body mass in (1239 ± 33 mg) by using a milk-extracted calcium powder (Capolac®, Arla Foods Ingredients amba, Denmark) and was therefore a dairy source rather than supplemental calcium carbonate. The source of calcium is an important consideration regarding the physiological response[8]. The calcium powder was completely soluble in milk and the quantities used to increase the calcium content of the meal resulted in negligible increases in protein, carbohydrate and fat (all < 0.5 g) and sodium, magnesium, chloride and potassium (all < 90 mg). As our working hypothesis suggests that dietary calcium influences gastrointestinal peptide secretion at the level of the gut, calcium absorption was not pertinent. The dose of calcium in CAL was chosen as an attempt to maximize the difference in the luminal concentration of calcium between CON and CAL (as a proof-of-concept), without exceeding the upper tolerable limit for adults[32].

Water consumption was *ad libitum* during the postprandial period in the first trial and replicated for the subsequent trial. A 180-min postprandial period started upon consumption of the first mouthful of breakfast, which was consumed within 5 min. Following this, participants ran on a treadmill for 60 min at a speed designed to elicit 60% of peak oxygen uptake, considered moderate-intensity[33].

Anthropometric measurements

Body mass was determined to the nearest 0.1 kg using balance scales (Seca, Birmingham, UK) upon arrival to the laboratory, where participants wore only light clothing. Height was measured to the nearest 0.1 cm using a stadiometer (Seca, Birmingham, UK).

Blood sampling and analysis

Blood samples were collected at baseline, and at 15, 30, 45, 60, 90, 120, 180 min following breakfast consumption. Additional blood was sampled at 20, 25, 35 and 40 min (when it was expected that gastrointestinal peptide and insulin concentrations would peak) in order to increase resolution of the postprandial AUC. Samples were obtained whilst participants were supine to control for posture-induced changes in plasma volume. A 20 µl capillary tube was filled with whole blood to determine glucose and lactate concentrations immediately using a glucose/lactate analyzer (Biosen C_line, EKF Diagnostics, Magdeberg, Germany). 10 ml of whole blood was allowed to stand for 30 min in a non-anticoagulant tube before being centrifuged at 3000 g and 4°C for 10 min. Aliquots of serum were stored at -80°C for later determination of insulin (IBL International, Hamburg, Germany) and NEFA (WAKO Diagnostics, Richmond, VA) concentrations in duplicate. Intra-assay coefficients of variation were 3.7 % and 5.7 % for insulin and NEFA assays, respectively.

Further, 4 ml EDTA tubes were filled, containing 200 kIU of aprotinin per ml of whole blood and were centrifuged immediately at 3000 g and 4°C. The supernatant was stored immediately at -80°C for later determination of GIP_{1-42} (Immuno-Biological Laboratories Co., Ltd, Japan) and total GLP-1 concentrations (Epitope Diagnostics, San Diego, CA).

Subjective ratings

Paper based, 100 mm VAS were completed at baseline, immediately following breakfast and every 30 min thereafter. Questions asked were used to determine hunger, fullness, satisfaction and prospective food consumption at all time points. These were also combined to give a combined-appetite score[34] where:

147 Combined-appetite score = [hunger + prospective food consumption + (100 – fullness) +
148 (100 – satisfaction)] / 4
149

150 The individual components (hunger, fullness, prospective consumption and satisfaction) were still presented
151 alongside the combined-appetite score, in order to discern the aspects of appetite that may have different
152 determinants. Fullness, for instance, may be more closely associated with peripheral physiological changes than
153 other aspects[35,36], which may additionally be influenced by emotional and environmental cues.
154 Immediately following breakfast consumption a further VAS was completed, whereby questions asked
155 were used to determine meal palatability, visual appeal, smell and taste. At the end of the trial participants were
156 also asked to indicate whether they believed they had consumed the CON or CAL meal to assess whether the
157 calcium could be detected.
158

159 **Energy expenditure and substrate oxidation**

160 Substrate metabolism was estimated with rates of oxygen consumption (VO_2) and carbon dioxide
161 production (VCO_2) using stoichiometric equations, and was adjusted during exercise to account for the
162 contribution of glycogen to metabolism[33]:
163

164 Rate of lipid oxidation at rest and during exercise (g/min) = (1.695 x VO_2) – (1.701 x
165 VCO_2)
166

167 Rate of carbohydrate oxidation at rest (g/min) = (4.585 x VCO_2) – (3.226 x VO_2)
168

169 Rate of carbohydrate oxidation during exercise (g/min) = (4.585 x VCO_2) – (2.962 x
170 VO_2)
171

172 (VO_2 and VCO_2 are L/min)
173

174 Energy expenditure was calculated based on lipids, glucose and glycogen providing 40.81, 15.64 and
175 17.36 kJ/g, respectively. At rest, calculations were based on glucose providing all the carbohydrate for

metabolism, whereas during moderate intensity exercise carbohydrate oxidation is met by glucose and glycogen providing 20 and 80% contributions, respectively[33].

Expired gas samples were collected using a breath-by-breath system (Metalyzer 3B, Cortex, Germany) calibrated using gases of known concentration and a 3 L syringe. For resting samples, participants wore a facemask and lay supine and after a 5-min stabilization phase, 10-min samples were obtained and averaged at baseline, and every 60 min after breakfast consumption in accordance with best practice methods[37]. Expired gas was continuously sampled throughout exercise and averaged over each 5-min period ignoring the first 5 min to allow for VO_2 and VCO_2 to reach a steady-state.

Statistical analysis

As we could not find data pertaining to either the expected effect size or the typical error of measurement for our primary outcomes of postprandial GIP_{1-42} or GLP-1 responses, we based our sample size on the insulin response. Using pilot data, a high-calcium meal resulted in a 12.9% increase in insulinemia (unpublished observation. Gonzalez JT, Rumbold PL, Stevenson EJ. 2012). With a typical error of 8.4% for postprandial insulinemia[38], 10 subjects should provide statistical power above 80% with an alpha level of 0.05.

Subjective appetite ratings and blood analyte concentrations were converted into time-averaged area under the curve (AUC) using the trapezoidal rule. As the time points after ingestion may influence the effect of a particular satiety related component (hormonal, metabolic, physical or cognitive[39]) the postprandial period was split into 0-60, 0-120 and 0-180 min.

Data were tested for normal distribution using the Anderson-Darling normality test and data not displaying normal distribution were log-transformed prior to statistical analysis.

Paired t-tests were used to determine differences at baseline, and differences in postprandial AUCs between trials.

To determine whether habitual calcium intake influenced the postprandial responses to calcium co-ingestion, pearson product-moment correlation coefficients were used to determine relationships between habitual calcium intake and the change in postprandial AUC of each of the variables.

Statistical significance was set at $P < 0.05$. All results are presented as mean ± SEM unless stated otherwise.

RESULTS

All participants completed all trials ($n = 10$), however due to difficulties with blood collection in one participant, data for GIP_{1-42}, GLP-1 and NEFA are presented from 9 participants. The participants age, height, body mass, BMI, peak oxygen uptake and habitual calcium intake were (mean ± SD) 25 ± 3 y, 178.3 ± 4.9 cm, 82.6 ± 6.9 kg, 26.0 ± 2.1 kg/m^2, 53.1 ± 4.1 ml/kg/min and 1084 ± 544 mg/d.

Plasma GIP_{1-42} and GLP-1 and serum insulin

No significant differences were observed for plasma GIP_{1-42} or GLP-1, or serum insulin concentrations at baseline ($P > 0.05$).

Postprandial GIP_{1-42} rose to a peak concentration of 27.5 ± 7.0 pmol/L in the CON trial and a significantly greater 47.7 ± 7.0 pmol/L in the CAL trial ($P = 0.028$; Figure 2A). The GIP_{1-42} postprandial AUC for 60, 120 and 180 min were 60, 47 and 43 % greater in the CAL trial, compared to CON, respectively (Table 1; all $P < 0.05$).

GLP-1 rose following breakfast consumption to peak concentrations of 5.2 ± 1.3 and 5.9 ± 1.3 pmol/L in CON and CAL trials, respectively ($P > 0.05$; Figure 2B). The GLP-1 AUC for 120 min post-breakfast was 22 % greater in CAL vs. CON (Table 1; $P = 0.047$).

Peak insulin concentrations tended to be greater following CAL vs. CON (445 ± 59 vs. 547 ± 72 pmol/L; $P = 0.063$; Figure 2C). The insulin AUC for the 120 min following breakfast consumption was 19% greater with CAL vs. CON ($P = 0.03$; Table 1).

Glucose, lactate and NEFA

There was no significant difference between trials in glucose or lactate or NEFA concentrations at baseline ($P > 0.05$).

No significant differences were detected for the glucose AUC (Figure 3A; Table 1), however, the lactate AUC for the first 60 min after meal consumption was significantly greater in CAL vs. CON (Figure 3B; Table 1; $P < 0.036$).

The AUC for NEFA were not significantly different between trials (Table 1). NEFA concentrations were maximally suppressed at ~30 min following breakfast consumption to 0.08 ± 0.01 and 0.08 ± 0.00 mmol/L before rising to 0.29 ± 0.05 and 0.33 ± 0.06 mmol/L at the end of the postprandial period in the CON and CAL trials, respectively. As such NEFA concentrations were virtually back to baseline values at the onset of exercise (Figure 3C).

Subjective ratings

The visual appeal (CON: 54 ± 7, CAL: 59 ± 7), smell (CON: 71 ± 5, CAL: 76 ± 3), taste (CON: 66 ± 6, CAL: 66 ± 7), and palatability (CON: 65 ± 7, CAL: 71 ± 7) of the breakfasts were not significantly different (all $P > 0.05$). Additionally, participants guessed the correct breakfast administered on 7 out of 20 occasions, which is below the 50% considered as random chance.

There were no significant differences in any appetite ratings at baseline (all $P > 0.05$). The satisfaction AUC for the first hour following breakfast consumption was 5 mm (10%) greater in the CAL trial compared to CON ($P = 0.036$).

The combined-appetite AUC for the first hour following consumption was 5 mm (12%) lower in the CAL trial compared to CON ($P = 0.034$; Table 2; Figure 4).

Energy expenditure and substrate utilisation

There was no difference in lipid oxidation, carbohydrate oxidation or energy expenditure at baseline (all $P > 0.05$). There was also no interaction effect for either lipid oxidation, carbohydrate oxidation or energy expenditure (all $P > 0.05$). Neither postprandial nor exercise substrate oxidation differed between trials (Table 3; all $P > 0.05$).

Correlations between variables

There were no significant relationships between habitual calcium intake and the change in the postprandial AUC of GIP_{1-42}, GLP-1, insulin or subjective appetite sensations in response to calcium co-ingestion (all $P > 0.05$).

DISCUSSION

The primary finding from this study is that calcium co-ingestion potentiates postprandial plasma glucose-dependent insulinotropic $peptide_{1-42}$ and glucagon-like peptide-1 concentrations in humans. Postprandial insulinemia and satiety were also increased with high-calcium ingestion. Substrate metabolism on the other hand, was unaffected by the calcium content of the meal.

High luminal calcium concentrations stimulate the secretion of GIP and $GLP\text{-}1_{7-36}$ by isolated rat intestine[25], probably acting via the calcium sensing receptor. Thus, we hypothesized that an increase in the calcium content of a meal would increase the calcium concentration that the K- and L-cells are exposed to, and thus potentiate postprandial GIP and GLP-1 concentrations. This is the first study in humans to show that increasing the calcium content of a meal (from ~250 mg to ~1240 mg) amplifies the 2 h postprandial responses of GIP_{1-42} and total GLP-1 (by 47% and 22%, respectively). Although we could not confirm that we were able to increase the calcium concentration in the intestine, this is a likely mechanism for the responses observed.

Lending support to this thesis, GIP_{1-42} concentrations were potentiated to a greater extent, and the enhancement was initiated more rapidly, than GLP-1 concentrations [GIP is secreted primarily from the more proximal duodenum[40], compared to the more distal jejunum and ileum for GLP-1[40] and postprandially, the duodenum is exposed to a higher concentration of calcium than the ileum[24]].

A second potential mechanism could be that decreased dietary fat absorption[7] resulted in a greater luminal fat content in the distal small intestine, thereby stimulating GLP-1 secretion in the later postprandial period, though this is does not explain the rapid changes in GIP_{1-42} concentrations. Calcium can also delay gastric emptying[41], and delayed gastric emptying can influence the gut peptide response[42]. However, it is just as plausible that the calcium induced increase in GLP-1, could drive the gastric emptying response[17] and hence, the direction of causality is currently unclear.

To the best of our knowledge, only one other study has determined GLP-1 responses to acute calcium ingestion[8], and found no significant effect on the postprandial AUC (GLP-1 was however, higher at 60 min following consumption of calcium carbonate). Although the large energy load (50% of total daily energy intake) provided, could have produced such a large hormonal perturbation that more subtle effects were masked. Others, using a smaller energy load determined insulin concentrations and appetite sensations following high-calcium meals[4,43], although by sampling at an hourly rate, the transient effects that are reported in the present study would have been missed.

CAL also increased insulinemia and influenced subjective appetite ratings in the postprandial period. It may be that increased GIP_{1-42} and GLP-1 concentrations are, in part, responsible for this. Both peptides potently stimulate insulin secretion when blood glucose concentrations are elevated[10]. The transient period with which insulin concentrations were potentiated imply that this is a glucose-dependent effect, as the amplification is only present when blood glucose concentrations were also above fasting values.

The lower combined-appetite score with CAL was a relatively small effect and short-lived. Moreover, the impact of these findings on subsequent energy intake should be interpreted with caution, as energy intake is determined primarily by the portion size served[39] in the "real-world" setting (ie. individuals consume most of what they are served, most of the time, regardless of appetite sensations or hormonal profile). Nonetheless, self-served portion size is influenced by the previous satiety experienced by food consumption[44]. Hence, an increase in satiety (decreased appetite) could have a modest effect on long-term energy balance. Moreover, a ~16% reduction in 24-h energy intake has been reported after a high-calcium meal[4].

It cannot be proved that the transient reduction in combined-appetite sensations with CAL was due to the changes in GLP-1 and insulin reported, particularly as other hormones, known to be influenced by calcium ingestion (such as calcitonin), are anorectic[45]. However, as serum calcitonin peaks at ~180 min following calcium ingestion[46], at which point appetite was similar between trials, it is unlikely to be a factor in the present study. In contrast, the time-course of gastrointestinal peptide, insulin and appetite responses all appear to be similar.

Acute insulin administration suppresses food intake in rodents[47], and is strongly associated with postprandial fullness sensations in humans[48,35]. In addition, $GLP\text{-}1_{7-36}$ infusion dose-dependently reduces appetite and food intake in humans[13]. Hence, insulin and GLP-1 both affect appetite directly (with GIP providing an indirect influence through insulin). The simultaneous effects, combined with the evidence from

311 infusion studies makes it tempting to speculate that these responses are linked, but this needs clarifying with
312 future work.

313 We chose to measure total GLP-1 rather than the "active" GLP-1_{7-36}. In humans, all the GLP-1 secreted
314 is in the 7-36 form[49]. Before entering the systemic circulation, approximately 50% has been cleaved [9]
315 producing GLP-$1_{9-36,}$ believed to be physiologically inactive [but may act through receptors other than the
316 classical GLP-1 receptor[50]]. However it is thought that GLP-1 can act centrally [acting on the hypothalamus
317 via sensory afferent neurons and subsequently neurons of the solitary tract nucleus [51]] prior to entering the
318 circulation, and potentiate insulinemia through neural and direct pancreatic β-cell stimulation[12,52]. Thus,
319 measuring the active form and its metabolite provides an indication of total GLP-1 secretion, which is likely to
320 have physiological effects before entering the circulation[9].

321 Contrary to GLP-1, GIP is thought to act only through its receptor as GIP_{1-42}[50] and as such, we chose
322 to measure GIP_{1-42} to reflect the tissue exposure to the active peptide. GIP_{1-42} is not believed to have a direct
323 impact on appetite, but could have an influence through GLP-1 and insulin secretion.

324 In spite of a 20% increase in insulinemia, we saw no difference in glycemia or serum NEFA
325 availability between trials. The reason for this is likely due to the glucagonotropic effect of GIP[10,53,54],
326 thereby maintaining the glucagon/insulin ratio. Furthermore, the greater insulinemia was transitory in nature
327 (with the greatest differences seen from 20-60 min postprandially), and this not of sufficient duration to
328 influence glucose uptake.

329 The similar lipid oxidation between trials support the findings of some[55,56] but not others[4,57].
330 When lipid oxidation has been stimulated by a high-calcium meal, this has occurred with simultaneous
331 enhanced NEFA availability[57,4]. We did not observe a difference in NEFA availability, and NEFA
332 concentrations had returned to almost fasting values prior to exercise. Therefore the meal-induced suppression
333 of NEFA concentrations[58] was not overriding the ability to detect a difference in either NEFA availability or
334 lipid oxidation. The discrepancy between studies may, in part, be accounted for by the populations used (healthy
335 BMI vs. overweight/obese) as metabolic functions of GLP-1, are more apparent in obesity[17,18].

336 Blood lactate concentrations were elevated with calcium co-ingestion for the first 60 min following
337 ingestion, which could indicate a greater reliance on carbohydrate oxidation. An explanation for this is not
338 readily forthcoming, particularly given that DPP-IV inhibition (which elevates GIP_{1-42} and GLP-1_{7-36}
339 concentrations) decreases muscle dialysate lactate concentration[19], although the 120 and 180 min postprandial

AUC were similar between trials.

The dose of calcium in the CAL trial was within the upper tolerable limit for adults[32], although would unlikely be consumed in a single-meal under normal-circumstances (this dose equates to almost 1L of whole milk). This may in part, explain the discrepancy between the present study and that of Lorenzen *et al.[8]* as the high calcium dose with a small meal in the present study would result in a higher concentration of calcium in the gastrointestinal tract, compared to a similar or smaller dose of calcium diluted in a large meal[8]. Furthermore the findings of this study should be placed into the context of clinical relevance. On average, the participants had an adequate habitual calcium intake and a normal BMI. Calcium supplementation at high levels has been implicated in renal stone formation[59] and cardiovascular disease mortality[60]. Although neither of these are without controversy[61,62]. Insulin *per se* can cause atherogenesis and insulin resistance, which in turn are strongly associated with cardiovascular disease (reviewed in[63]). Thus raising the question of the cost/benefit of the insulin and gut peptide responses seen in the present study, particularly if this is also seen in high-risk populations.

A limitation with this study is the relatively small sample size, and thus the relevance to a wider population needs evaluating. This study does however, provide a proof-of-principle that calcium ingestion influences postprandial insulin and gastrointestinal peptide concentrations and appetite sensations, and further work should aim to establish the dose-response of this relationship.

It is noteworthy that apart from the calcium content, the test meals were identical in nutritional composition and similar in palatability, visual appeal, taste and smell. Participants were also unable to detect a difference between the test meals and as such the impact of palatability on postprandial appetite[64] or insulinemia[65] can be eliminated.

In conclusion, increasing the calcium content of a meal augments postprandial circulating GIP_{1-42} and total GLP-1 concentrations in humans. The high-calcium meals also resulted in greater insulinemia and satiety. Substrate metabolism, however, was not affect by calcium co-ingestion.

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and EJS wrote the paper; JTG had primary responsibility for final content. All authors read and approved the final manuscript. None of the authors had a personal or financial conflict of interest.

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TABLE 1

Postprandial responses of circulating parameters to calcium co-ingestion in young healthy males

	Time-averaged postprandial area under the curve					
	0-60 min		0-120 min		0-180 min	
Variable	CON	CAL	CON	CAL	CON	CAL
Plasma GIP_{1-42} (pmol/L)	21 ± 6	33* ± 6	18 ± 5	25* ± 5	13 ± 3	18* ± 3
Plasma GLP-1 (pmol/L)	3.18 ± 0.75	3.96 ± 1.17	2.72 ± 0.62	3.71* ± 1.26	2.43 ± 0.56	3.41 ± 1.24
Serum insulin (pmol/L)	235 ± 24	278 ± 27	161 ± 12	192* ± 15	133 ± 10	161 ± 13
Blood glucose (mmol/L)	5.6 ± 0.2	5.7 ± 0.2	5.1 ± 0.1	5.2 ± 0.1	4.9 ± 0.1	5.0 ± 0.1
Blood lactate (mmol/L)	0.73 ± 0.05	0.90* ± 0.06	0.75 ± 0.05	0.86 ± 0.07	0.73 ± 0.06	0.79 ± 0.06
Serum NEFA (mmol/L)	0.18 ± 0.02	0.21 ± 0.03	0.15 ± 0.02	0.16 ± 0.02	0.17 ± 0.02	0.19 ± 0.02

Data are expressed as means ± SEM. Data not normally distributed as assessed by the Anderson-Darling normality test were log-transformed for statistical analysis. CON, control; CAL, calcium co-ingestion; GIP_{1-42}, glucose-dependent insulinotropic $peptide_{1-42}$; GLP-1, glucagon-like peptide-1; NEFA, non-esterified fatty acid. *Significant difference between CAL vs. CON, $P < 0.05$ assessed by paired t-tests.

TABLE 2

Postprandial subjective appetite responses to calcium co-ingestion in young healthy males

	Time-averaged postprandial area under the curve					
	0-60 min		0-120 min		0-180 min	
Variable	CON	CAL	CON	CAL	CON	CAL
Hunger	40 ± 6	36 ± 6	43 ± 6	41 ± 6	47 ± 6	46 ± 5
Fullness	56 ± 7	60 ± 7	51 ± 6	53 ± 6	45 ± 6	49 ± 6
Satisfaction	54 ± 6	59* ± 6	50 ± 6	52 ± 6	45 ± 6	47 ± 6
Prospective consumption	47 ± 7	40 ± 7	50 ± 7	47 ± 6	54 ± 6	52 ± 6
Combined-appetite	44 ± 6	39* ± 6	48 ± 6	46 ± 6	53 ± 6	51 ± 6

Data are expressed as means ± SEM. CON, control; CAL, calcium co-ingestion. *Significant difference between CAL vs. CON, $P < 0.05$ assessed by paired t-tests.

TABLE 3

Substrate oxidation and energy expenditure during the postprandial and exercise periods

	Postprandial period (0-180 min)		Exercise period (180-240 min)	
Variable	CON	CAL	CON	CAL
Lipid oxidation (g)	12.2 ± 0.6	13.1 ± 0.5	27.3 ± 1.1	29.1 ± 1.0
CHO oxidation (g)	42.7 ± 1.5	38.6 ± 1.3	129.0 ± 3.4	121.2 ± 4.0
Energy expenditure (kJ)	1168 ± 17	1139 ± 20	3135 ± 34	3085 ± 33
RER (au)	0.870 ± 0.01	0.856 ± 0.004	0.894 ± 0.004	0.883 ± 0.005

Data are expressed as means ± SEM. CON, control; CAL, calcium co-ingestion; CHO, carbohydrate; RER, respiratory exchange ratio. No significant differences were detected between trials.

Figure Legends:

FIGURE 1. Schematic representation of the main trials.

FIGURE 2. Plasma GIP_{1-42} (A) and GLP-1 (B), and serum insulin (C) concentrations following consumption of a control (CON; 3 mg calcium/kg body mass) or high-calcium (CAL; 15 mg calcium/kg body mass) meal. Data are means ± SEM, n = 9. GIP_{1-42}, glucose-dependent insulinotropic $peptide_{1-42}$; GLP-1, glucagon-like peptide-1; grey rectangle represents the exercise period.

FIGURE 3. Blood glucose (A), lactate (B), and serum NEFA (C) concentrations following consumption of a control (CON; 3 mg calcium/kg body mass) or high-calcium (CAL; 15 mg calcium/kg body mass) meal. Data are means ± SEM, n = 10 for glucose and lactate and n = 9 for NEFA. Grey rectangle represents the exercise period.

FIGURE 4. Combined-appetite scores following consumption of a control (CON; 3 mg calcium/kg body mass) or high-calcium (CAL; 15 mg calcium/kg body mass) meal. Data are mean ± SEM, n = 10. Grey rectangle represents the exercise period.